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RESEARCH ARTICLE

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A newly discovered interference of the central nitrergic system on oxytocin-induced hypophagia in layer-type chickens

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ABSTRACT

Various neurochemical pathways are participating in the regulation of food intake in mammals and birds. Both oxytocin (OT) and nitric oxide (NO) are known as hypophagic agents in birds. This study consisted of 6 experiments and each experiment had 4 groups (ngroup=11, 5-day-old chickens). In all experiments, 3-hour food-deprived (FD3) birds received intracerebroventricular (ICV) injections either control diluent or drug solution. Then the birds had ad libitum access to the food and fresh water and then cumulative food intake (gr) was measured based on the percentage of the body weight (%BW). In experiments 1 to 3, ICV injections of L-arginine (precursor of NO, 200, 400, and 800 nmol), L-NAME (NOS inhibitor, 100, 200, and 400 nmol) and OT (2.5, 5, and 10 µg) were performed respectively. In experiment 4, each group received any ICV injections of L-NAME (100 nmol), OT (10 µg) or a co-injection of L-NAME (100 nmol) and OT (10 µg). In experiment 5, L-arginine (ICV, 200 nmol), OT (10 µg), or L-arginine (200 nmol) and OT (10 µg) were injected to the groups. Experiment 6 was similar to the experiment 5, although the dose of OT was 2.5 µg in all the treatment groups. Results showed that the ICV injection of L-NAME (100 nmol) significantly attenuated hypophagic effect induced by OT (10 µg) ($p < 0.05$). Findings suggested that NO might mediate the hypophagic effect of OT in FD3 neonatal layer-type chickens.

Keywords

L-NAME; L-arginine; food intake; bird

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Abbreviations

%BW: percentage of the body weight
FD3: 3-hour food-deprived
ICV: intracerebroventricular
L-NAME: N(G)-Nitro-L-arginine methyl ester
NO: nitric oxide
NOS: nitric oxide synthase
OT: oxytocin
OXTR: oxytocin receptor

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Introduction

Various neurotransmitters play a role in the control of food intake via activation of different neurochemical pathways inside the central nervous system (CNS). Within the last few decades, physiologists have discovered many neurochemical pathways that control the feeding behaviors and have tried to find out the possible interactions among them [1–3]. Nitric oxide (NO) is a free radical gaseous molecule produced from L-arginine by the action of nitric oxide synthase (NOS). Neurons, glia and vascular cells can express NOS and produce NO inside the brain [4]. NOS-containing neurons located in the hypothalamus primarily are presented in the paraventricular nucleus of hypothalamus (PVN) and supraoptic nucleus (SON). Also, the axons of these neurons project to the pituitary gland [5]. NO has different physiological functions in the CNS including regulation of pain, memory, learning, neurotransmitter release, and feeding behavior in mammals and birds [3,6–8]. Previous studies have shown that the ICV injection of L-arginine (400 and 800 nmol) as a precursor of NO, significantly reduced food intake in neonatal chickens. On the other hand, ICV administration of NG-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, increased food intake in neonatal layer-type chicken [8,9]. Although, NO is known as a feeding-inhibitory molecule in layer-type chicken, but ICV injection of NO has feeding-stimulatory effect in mice [10] and broiler-type chicken [11]. These divergent reports may relate to genetic differences between mammals and birds or even the different genetic background in between the strains. Other studies have shown that the effects of NO on food intake are mediated by other neurotransmitters. For instance, ICV injection of L-NAME attenuated the anorexigenic effect of corticotropin-releasing hormone (CRH) in neonatal chickens, while Neuropeptide Y (NPY)-induced feeding behavior was not affected by L-NAME [12]. Furthermore, the mediatory role of NO on hypophagia induced by leptin has been reported in broilers and Leghorns [13].

Oxytocin (OT) is known as a nano-peptide neurotransmitter and is associated with parturition, lactation, cognition, tolerance, adaptation, and complex sexual and maternal behaviors [14]. Besides, OT has a role in the regulation of food intake in mammals and birds [15–17]. OXTR belongs to G protein-coupled group and primarily pairing with Gq proteins to phospholipase C [18]. OXTR is highly expressed in the regions that are involved in food intake regulation, such as the hypothalamus, nucleus accumbens, amygdala, ventral tegmental area, frontal cortex, insula, and hindbrain [19,20]. The OT-like neurohypophysial hormone has been identified in non-mammalian vertebrates such as birds and frogs called mesotocin (MT). Studies presented structural and functional similarities between mesotocin and mammalian oxytocin and showed that avian mesotocin receptor (MTR) is orthologous to mammalian oxytocin receptor (OXTR) [21]. Jonaidi et al. (2003) reported that OT plays a unique role in reducing feed intake by acting on mesotocin (MT) and/or vasotocin receptors in chickens [17]. The previously same stimulating activity of both MT/OT about ACTH release in the hypothalamic–pituitary–adrenal axis of birds has been confirmed [22]. Similar to the effect of OT in mammals [23], several investigations have shown the dose-dependent hypophagic effect of this neurotransmitter in birds [17,24].

The presence of the NOS in the magnocellular neurons in the brain suggests the mediatory role of NO in the production and release of OT. Also, several studies showed that ICV injection of NO donors significantly mediates OT production and release in laboratory animals [25,26]. For example, the recent research on rats showed that penile erection induced by OT significantly decreased after the injection of SMTC, an inhibitor of the neuronal NOS, into the bed nucleus of the stria terminalis (BNST). Based on this evidence, OT induces this physiologic behavior via the mediatory role of NO [27]. In another study, the analgesic effect of OT in mice was investigated and the mediatory action of NO was illustrated by the injection of L-arginine inside the spinal cord [28]. All of this evidence highlights the mediatory role of NO on OT-induced behaviors in the CNS. In addition, the anatomical relationship between NOS-containing neurons and oxytocinergic neurons [29] boosting the possible interaction between central nitrergic and oxytocinergic systems in birds. Nevertheless, no evidence has been reported so far about the interconnection of these two systems on food intake in chickens. To test this hypothesis, 6 experiments were performed on 3-hour food-deprived (FD3) neonatal layer chicken to find out the probable interaction of OT and NO on food intake behavior.

Results

The possible interaction between nitrergic and oxytocinergic systems on cumulative food intake in FD3 neonatal layer-type chickens was illustrated in "Fig. 1-6". In experiment 1, the ICV injection of 200 nmol L-arginine had no significant effect on cumulative food intake in comparison with the control group in 30, 60, and 120 minutes post-injection ($p \geq 0.05$). While the ICV administration of 400 and 800 nmol L-arginine significantly and dose-dependently decreased the food intake in comparison to the control group in all the time-points ($p < 0.05$). These results suggest the dose-dependent hypophagic effect of NO in neonatal layer-type chicken [Treatment effect: F (3, 40) = 1749.01, $p < 0.01$; time effect: F (2, 80) = 4813.53, $p < 0.01$; treatment × time interaction: F (6, 80) = 29.17; $p < 0.01$] (Fig. 1).

In experiment 2, the ICV injection of 100 nmol L-NAME made no significant changes in cumulative food intake in comparison with the control group in 30, 60, and 120 minutes post-injection ($p \geq 0.05$). However, ICV injection of 200 nmol and 400 nmol L-NAME significantly enhanced cumulative food intake in comparison to the control group in all the time-points ($p < 0.05$). This data shows the hypophagic effect of NO in neonatal layer-type chicken due to the administration of the NOS inhibitor (L-NAME) [Treatment effect: F (3, 40) = 2841.72, $p < 0.01$; time effect: F (2, 80) = 5825.12, $p < 0.01$; treatment × time interaction: F (6, 80) = 52.81; $p < 0.01$] (Fig. 2).

In experiment 3, the ICV injection of 2.5 µg Oxytocin did not significantly alter the cumulative food intake in comparison with the control group in 30, 60, and 120 minutes post-injection ($p \geq 0.05$). However, the ICV administration of higher doses of Oxytocin, 5 µg, and 10 µg, significantly decreased cumulative food intake in comparison with the control group in all the time-points ($p < 0.05$). These results illustrate the dose-dependent hypophagic effect of Oxytocin in neonatal layer-type chickens [Treatment effect: F (3, 40) = 1038.46, $p < 0.01$; time effect: F (2, 80) = 3274.82, $p < 0.01$; treatment × time interaction: F (6, 80) = 21.65; $p < 0.01$] (Fig. 3).

In experiment 4, ICV co-injection of 100 nmol L-NAME and 10 µg of oxytocin, significantly attenuated the hypophagic effect of oxytocin in 30, 60, and 120 minutes post-injection ($p < 0.05$). Despite, 100 nmol of L-NAME had no significant effect on cumulative food intake in comparison with the control group in all the time-points ($p \geq 0.05$). This data may reveal the mediatory effect of nitrergic system on the hypophagic effect of OT in neonatal layer-type chickens [Treatment effect: F (3, 40) = 3482.73, $p < 0.01$; time effect: F (2, 80) = 5037.12, $p < 0.01$; treatment × time interaction: F (6, 80) = 41.53; $p < 0.01$] (Fig. 4).

In experiment 5, ICV co-injection of 200 nmol L-arginine and 10 µg of oxytocin, significantly amplified the hypophagic effect of oxytocin in 30, 60, and 120 minutes post-injection ($p < 0.05$). However, the ICV injection of L-arginine (200 nmol) could not significantly change cumulative food intake in comparison with the control group in all the time points ($p \geq 0.05$). This data support the possible interaction between nitrergic and oxytocinergic systems on food intake and nitrergic system may have a synergistic effect on the regulation of food intake induced by oxytocin in layer-type chickens [Treatment effect: F (3, 40) = 2538.47, $p < 0.01$; time effect: F (2, 80) = 4281.06, $p < 0.01$; treatment × time interaction: F (6, 80) = 37.26; $p < 0.01$] (Fig. 5).

In the final experiment, experiment 6, neither ICV injection of 200 nmol L-arginine nor administration of 2.5 µg oxytocin could significantly alter the cumulative food intake in comparison with the control group in 30, 60, and 120 minutes post-injection ($p \geq 0.05$). While the co-administration of both drugs significantly decreased the cumulative food intake in comparison with the control group in all the time-points ($p < 0.05$). Based on these results, a synergistic collaboration between these two central systems on the regulation of food intake in layer-type chicken

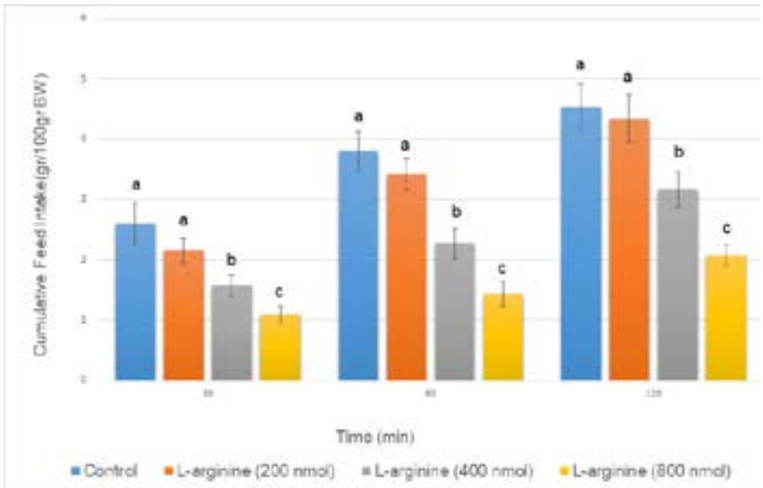

Figure 1.
Effect of ICV injection of L-arginine (200, 400 and 800 nmol) on cumulative food intake (% BW) in neonatal chickens is presented in mean ± SEM. There are significant differences between groups with different superscripts in a column (a, b and c; $p < 0.05$). L-arginine: precursor of NO

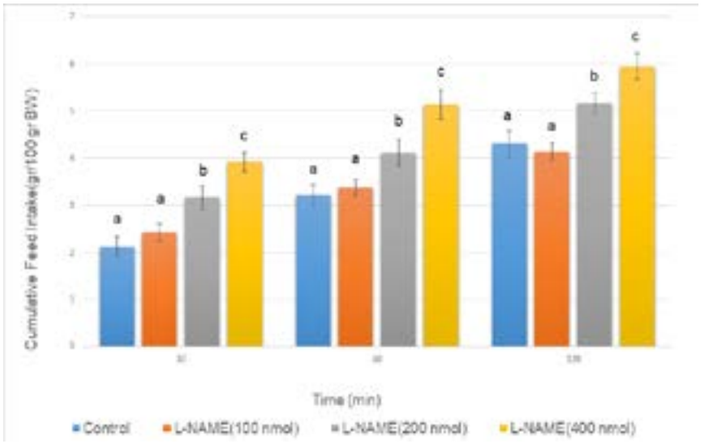

Figure 2.
Effect of ICV injection of L-NAME (100, 200 and 400 nmol) on cumulative food intake (% BW) in neonatal chickens is presented in mean ± SEM. There are significant differences between groups with different superscripts in a column (a, b and c; $p < 0.05$). L-NAME: NOS enzyme inhibitor

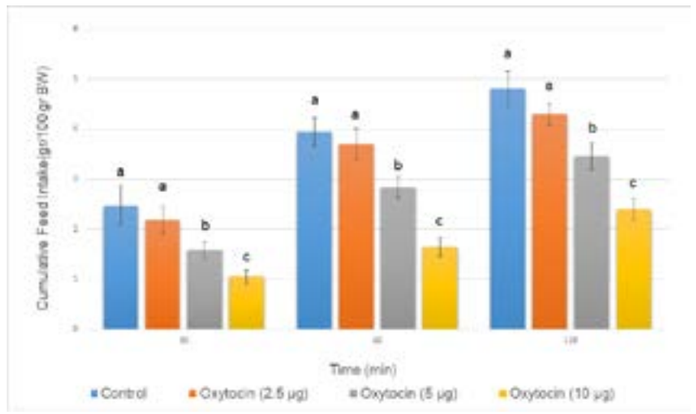

Figure 3.
Effect of ICV injection of oxytocin (2.5, 5 and 10 µg) on cumulative food intake (% BW) in neonatal chickens is presented in mean ± SEM. There are significant differences between groups with different superscripts in a column (a, b and c; $p < 0.05$)

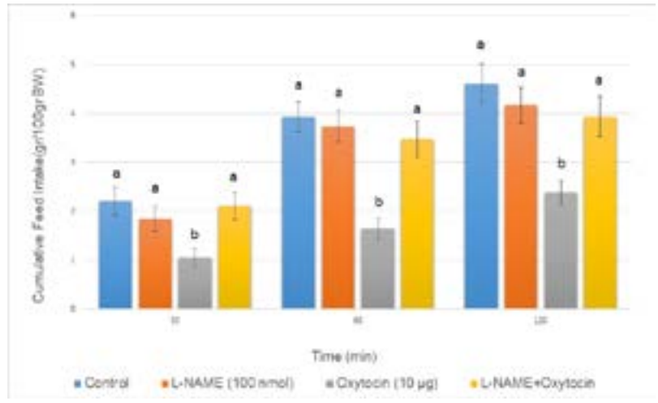

Figure 4.
Effect of ICV injection of L-NAME (100 nmol), oxytocin (10 µg) and their co-injection on cumulative food intake (% BW) in neonatal chickens is presented in mean ± SEM. There are significant differences between groups with different superscripts in a column (a and b; $p < 0.05$). L-NAME: NOS enzyme inhibitor

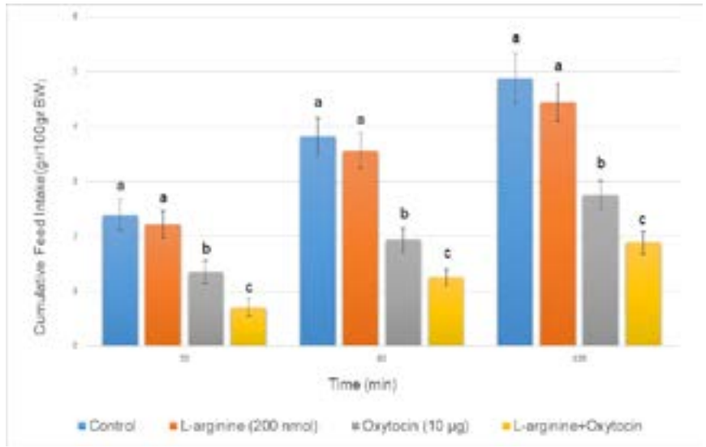

Figure 5.
Effect of ICV injection of L-arginine (200 nmol), oxytocin (10 µg) and their co-injection on cumulative food intake (% BW) in neonatal chickens is presented in mean ± SEM. There are significant differences between groups with different superscripts in a column (a, b and c; $p < 0.05$). L-arginine: NO precursor

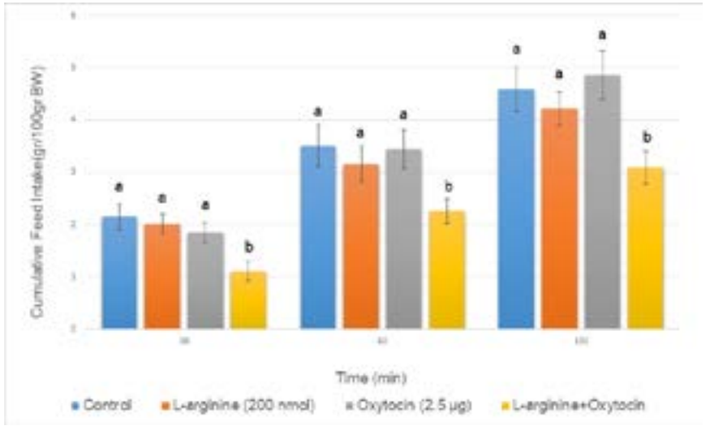

Figure 6.
Effect of ICV injection of L-arginine (200 nmol), oxytocin (2.5 µg) and their co-injection on cumulative food intake (% BW) in neonatal chickens is presented in mean ± SEM. There are significant differences between groups with different superscripts in a column (a and b; $p < 0.05$). L-arginine: NO precursor

is possible [Treatment effect: F (3, 40) = 1964.51, $p < 0.01$; time effect: F (2, 80) = 3275.83, $p < 0.01$; treatment × time interaction: F (6, 80) = 28.31; $p < 0.01$] (Fig. 6).

Discussion

This is the first report about the interaction between nitrergic and oxytocinergic systems on the regulation of food intake in FD3 neonatal layer-type chicken. Based on the results of this study, the ICV injection of L-arginine decreased the cumulative food intake in FD3 neonatal layers (Fig. 1). While the ICV administration of L-NAME increased the cumulative food intake (Fig. 2). Notably, some previous studies indicate an inconsistent finding regarding the effect of nitrergic system on food intake in layer-type and broiler-type chickens. For example, there is evidence that showed the intraperitoneal (IP) injection of L-NAME decreased feeding behavior in both layers and broilers [8], and the same has been recorded for IP injection of L-NAME in the rat [39]. The main reason for this discrepancy is probably due to the different routes of the administration, which could cause the involvement of peripheral NO receptors in the IP administration and consequently activation of different cascades on the food intake regulation. Choi et al. (1995) showed that the ICV injection of L-NAME had a hypophagic effect in broilers which is contrary to our findings [40]. On the other hand, the result of Khan et al. (2007) indicated that the ICV injection of L-NAME

increased food intake in layer-type chickens which is in agreement with our findings [8]. Another study revealed that the ICV injection of L-NG-Nitroarginine (L-NNA) a competitive NOS inhibitor, significantly diminished food intake in both broiler and Leghorn chicken which is in disagreement with our findings [13]. However, the other study about the interaction of nitrergic and cannabinoidergic systems showed the hypophagic effect of NO in neonatal layer-type chicken which is in agreement with the result of this study [38]. These opposite findings in layer-type and broiler-type chicken may be due to the different genetic characteristics between these strains. Additionally, different neurochemical pathways in feeding behavior are impressed by the different genetic backgrounds that eventually could cause even an opposite feeding response to the same neurotransmitters/neuromodulator [3,8,41–43].

OT is known as a regulator of food and water intake in mammals and OXTR(s) has been identified in the SON and PVN [15]. Also, this neurohormone is highly expressed in magnocellular neurons of the hypothalamus. It has been shown that both central and peripheral injections of OT have decreased food intake in mammals [18]. The hypophagic effect of OT in mammalian for the first time has been reported in the rat [15]. Kook et al. (1964) for the first time reported that the metabolic effects of OT in the chicken are the increased percentage of glucose and fatty acid in plasma and he showed that the responsive receptors to OT are expressed in the CNS of the bird those later has been discovered and called MTR by scientists as OXTR homologous [44]. Besides for the first time, Jonaidi et al. (2003) showed that ICV injection of OT could dose-dependently decreased feeding in meat-type chickens [17]. Furthermore, Mirnaghizadeh et al. (2017) confirmed the hypophagic effect of OT in neonatal broiler-type chickens [24]. These studies are in accordance with our results which presented the hypophagic effect of OT instead of MT on food intake in FD3 neonatal layer-type chicken again.

Several suggestions for the underlying mechanism of OT-induced hypophagia in mammals have been proposed. For instance, after ICV injection of OT, PVN neurons modulate intrinsic brainstem reflexes and directly control the vagal efferent projections toward the gastrointestinal system to inhibit gastric motility and ingestion [18]. Another suggestion is again related to PVN neurons, especially OT projections associated with melanocortin-4 receptors (Mc4R s) and they are a key point in food intake regulation [45,46]. The inhibitory effect of hypothalamic pro-opiomelanocortin (POMC) neurons and melanocortin-3/4 receptors on food intake have also been demonstrated in birds [47,48]. Nevertheless, the exact underlying mechanism of OT on food intake regulation in chicken has not been elucidated yet, however, OT or MT in birds may regulate food intake via similar pathways as in mammals.

Based on the literature we suppose the mediatory role of NO about food intake-induced by OT in chickens. The results of our study showed that NO has a mediatory effect on the hypophagic effect of OT in layer-type chickens and it has been reported that following the injection of NO donors and L-NAME into the lateral ventricle of the brain, release of the other neurotransmitters are altered in the different nuclei of the hypothalamus, especially in magnocellular neurons, which are involved in OT secretion. The presence of the NOS in the circumventricular organs and magnocellular neurons supports our results about the modulatory effect of NO on OT [49]. It is also suggested that NO increases the outputs of PVN and SON that can increase OT secretion in magnocellular neurons and ultimately modulate OT-physiological behaviors. This could be a possible explanation for the mediatory role of NO on the hypophagic effect of OT and that seems it is a synergistic one.

PVH also contains PVH containing nitric oxide synthase-1 (Nos1PVH) which is projected to the spinal cord and hindbrain and is involved in feeding behavior. It might be considered that OT-induced hypophagia is modulated by signaling of the Nos1PVH neurons in the brain. Also, several neuropharmacological studies indicated that PVH contains Sim1-expressing cell type (Sim1PVH) which is playing an important role in the control of food intake. In addition, Nos1PVH neurons are a subset of Sim1PVH neurons and oxytocin-expressing PVH neurons (OXTPVH) known as a subset of Nos1PVH neurons [50–52]. This evidence can be another clue to support our finding regarding the possible interaction between nitrergic and oxytocinergic systems in control of food intake in birds such as what has been already identified in mammalian.

Besides, the mediatory role of NO has been reported in negative inotropic and chronotropic effects induced by OT in the heart, and blockage of the NOS by systemic administration of L-NAME could decrease the protective effect of OT on myocardial cell [53,54]. Gutkowska and Jankowski (2009) mentioned that the cardioprotection effect of OT is dependent on the activation of intrinsic cardiac cholinergic neurons and NO release is the major underlying mechanism [55]. This finding indicated the mediatory role of NO on OT outside of the CNS. On the contrary, Reis et al. (2007) illustrated that endogenous NO could act as an inhibitory effect on OT secretion to keep fluid homeostasis of the body in mammals [25]. This inconsistency might be due to OT release following the ICV injection of angiotensin II and turning on another neural pathway, which is before NO, and this could alter the modulatory response of NO on OT release. Another study regarding the interaction of nitrergic and oxytocinergic systems outside of the CNS showed that the protective effect of OT in renal and hepatic injury induced by renal ischemia/perfusion in the adult male albino rat is probably NO-dependent. In this study administration of L-NAME before OT partially reversed the protective effect of OT and it seems that the protective effect of OT is dependent on NO production [56].

OT stimulates NOS and increases NO release in the CNS [5]. Also, ICV administration of OT enhanced NO and dopamine production via activation of neural pathways in the PVN and eventually related behavioral responses [27]. Canteros et al. (1995) suggested that increasing intracellular ionized Calcium and formation of Ca2+-calmodulin complex is the mechanism underlying activation of OT neurons in the hypothalamus and later this could activate the neural nitrergic system [57]. So far, the result of our study and other evidence are suggesting that oxytocin regulates NO release by stimulating NOS activity in the hypothalamus, while the oxytocinergic system is modulated by the nitrergic system. To support this finding, Nomura et al. (2005) showed the involvement of neuronal NOS-derived in the regulation of oxytocin gene expression in the hypothalamus [58]. Also, the up-regulation of NOS-mRNA by oxytocinergic neurons was demonstrated in the hypothalamo-neurohypophysial system [59]. These studies are supporting our findings and plotting a possible mechanism for the interaction of these two systems, although further investigations to elucidate the exact underlying signaling mechanism in birds are still needed.

Furthermore, some studies have shown that sexual and hormonal functions of OT in the hypothalamus such as regulation of the preovulatory gonadotropin-releasing hormone (GnRH) surge is a NO-dependent mechanism and can be amplified by injection of NO donors [60,61]. These results are in agreement with the results of our study and this could be considered as another clue to support our hypothesis about the possible interaction between these two systems.

We have observed functional synergistic interaction between nitrergic and oxytocinergic systems on the regulation of food intake in FD3 neonatal layer-type chickens and we found that OT-induced hypophagia is NO-dependent in the neonatal layers.

Materials & Methods

Animals

In order to investigate the possible interaction between NO and OT in the regulation of food intake, female one-day-old layer-type birds were bought from a local hatchery (Morghak Company, located in Tehran, Iran, n=264) and they were kept together for two days. After this, the chickens were moved into the individual cages randomly. Cage temperature and relative humidity were maintained at 32 C ± 1 (Electrical heat) and 40–50 % respectively. The lighting/dark phase was 23:1 hr [30,31]. All birds throughout this study had free access to a commercial starter diet and freshwater. It was consisting of 21% crude protein and 2850 kcal/kg metabolizable energy (Animal Science Research Institute Co. Iran, Table 1). The chicken was deprived of food for three hours (FD3) before any ICV injection, however, freshwater was available all the time. The initiation of the ICV injection was when the chickens were five-day-old. All the procedures were performed according to the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health, USA (publication No. 85-23, revised 1996) and were approved by the Institutional Animal Ethics Committee of Faculty of Veterinary Medicine, University of Tehran.

Experimental drugs

All of the experimental drugs including L-arginine (precursor of NO), Oxytocin (OT), N(G)-Nitro-L-arginine methyl ester (L-NAME, nitric oxide synthesis inhibitor) and Evans blue were bought from Sigma Co. (Sigma, USA). All drugs were dissolved in absolute dimethyl sulfoxide (DMSO) initially and later were diluted with 0.85 % saline containing Evans blue at a ratio of 1:250.

ICV injection procedures

Six experiments were designed and each one had four groups (A–D) (ngroup=11). Chickens were assigned into the groups based on their body weights (scale GF-6100, Japan accuracy = 0.01g). Therefore, maximum consistency was achieved based on the bodyweight in the groups. In every experiment, the non-anesthetized birds were injected intracerebroventricularly by a microsyringe (Hamilton, Switzerland) only once [32,33]. In this technique, the bird's head was supported with an acrylic device in which the bill holder of the device made a 45° angle with the table and the scalp was parallel to the tabletop [34]. A plate with a fenestra was immediately located above the skull over the right lateral ventricle. A microsyringe was inserted into the right ventricle via the fenestra and the tip of the needle pierced just 4 mm below the skull skin and the solution was discharged through the ventricular fluid gradually (volume of each injection was 10µL). Notably, this route of injection causes no physiologic stress in the neonatal birds [35]. Evans blue was added into all injections and at the end of the experiment, chicken was decapitated immediately and by observing the blue color inside the ventricles the corrected ICV injection was confirmed. Data for statistical analysis was only obtained from those individuals who had the correct injection (n correct injection = 9-11) [36,37].

Food intake measurement protocol

In experiment 1, chickens in the group (A) received ICV injection of control solution and in the group (B), (C) and (D), they were administered by 200, 400, and 800 nmol of L-arginine respectively. Experiments 2 and 3 were conducted similarly to experiment 1, however, in experiment 2, the different doses of L-NAME 100, 200, and 400 nmol and experiment 3, the different

doses of OT 2.5, 5, and 10 µg were injected into the lateral ventricle respectively. In experiment 4, chickens in the group (A) were received ICV injection of control solution, group (B) was injected with L-NAME (100 nmol), group (C) was administered with Oxytocin (10 µg) and group (D) received a co-injection of L-NAME (100 nmol) and oxytocin (10 µg). Experiment 5 was conducted similarly to experiment 4, except L-arginine (200 nmol) was used instead of L-NAME (100 nmol) in group (B) and group (D) received the co-injection of L-arginine (200 nmol) and oxytocin (10 µg). Experiment 6 was conducted similarly to experiment 5 and only a different dose of oxytocin (2.5 µg) was administered in groups (C) and (D) (Table 2). Immediately after the injection, chickens were returned to their individual cages and ad libitum food (pre-weighed) and water were provided. Then, the cumulative food intake was recorded at 30, 60, and 120 minutes post-injection by reweighting of the food. In order to decrease the effect of the bodyweight on the food intake volume, the food intake calculation was based on the bodyweight percentage (% BW). Following Jonaidi et al. (2003) study and based on our latest published articles about hypophagic role of OT in chicken [24,38], we have used OT instead of MT and the suggested doses to stimulate related receptors inside the brain.

Statistical analyses

Cumulative food intake was analyzed by repeated measure two-way analysis of variance (ANOVA) and is presented as the mean ± SEM. For treatments found to affect according to the ANOVA, mean values were compared with the Bonferroni test. p values < 0.05 were considered to indicate significant differences between treatment groups. The analysis of variance was performed using the model as given: $Yijk= \mu + \alpha j + \beta k + (\alpha\beta)jk + \varepsilon ijk$ Where Yijk is the value of its individual observation for valuables, µ: the grand mean, αj: is the treatment effect for the time, βk: is the treatment effect for the drugs, (αβ)jk: is the interaction effect for the time and drugs, εijk: error.

Table 1.
Ingredient and nutrient analysis of the experimental diet

Ingredient	(g/kg)	Nutrient analysis	(g/kg)
Maize	528.5	ME, MJ/kg	11.9
Soybean meal, 48% CP	315.7	Crude protein	210
wheat	50	Linoleic acid	17
Gluten meal, 61% CP	25.0	Crude fiber	36
Wheat bran	24.7	Calcium	10
Di-calcium phosphate	19.2	Available phosphorus	5
Oyster shell	12.3	Sodium	1.5
Soybean oil	10.0	Potassium	9.6
Mineral premix	2.5	Chlorine	1.7
Vitamin Premix	2.5	Choline	13012
Sodium bicarbonate	2.1	Arginine	11.4
Sodium chloride	2.0	Isoleucine	7.3
Acidifier	1.5	Lysine	12.1
DL-Methionine	1.0	Methionine	4.9
Toxin binder	1.0	Methionine + Cystine	8.3
L-Lysine Hcl	0.5	Threonine	7.0
cholecalciferol	1.0	Tryptophan	2.0
Multi enzyme	0.5	Valine	7.8

ME: metabolizable energy, CP: crude protein, per kg of diet, the mineral supplement contains 35.2 g manganese from $MnSO_4.H_2O$; 22g iron from $FeSO_4H_2O$; 35.2 g zinc from ZnO; 4.4 g copper from $CuSO_4.5H_2O$; 0.68 g iodine from ethylenediamine dihydroiodide; 0.12 g selenium from Na_2SeO_3. The vitamin supplement contains 1.188 g of retinyl acetate, 0.033 g of DL-α-tocopherol, 1.32 g of menadione, 0.88 g of thiamine, 2.64 g of riboflavin, 13.2 g of nicotinic acid, 4.4 g of pantothenic acid, 1.76 g of pyridoxine, 0.022 g of biotin, 0.36 g of folic acid and 1500 mg of choline chloride.

Table 2.
Intracerebroventricular injections in experiments

group	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6
A	CS*	CS	CS	CS	CS	CS
B	L-arginine[¡]	L-NAME[¥]	OT[§]	L-NAME	L-arginine	L-arginine
	(200 nmol)	(100 nmol)	(2.5 µg)	(100 nmol)	(200 nmol)	(200 nmol)
C	L-arginine	L-NAME	OT	OT	OT	OT
	(400 nmol)	(200 nmol)	(5 µg)	(10 µg)	(10 µg)	(2.5 µg)
D	L-arginine	L-NAME	OT	L-NAME+OT	L-arginine+OT	L-arginine+OT
	(800 nmol)	(400 nmol)	(10 µg)	(100 nmol)+(10 µg)	(200 nmol)+(10 µg)	(200 nmol)+(5 µg)

*CS: Control solution
[¥]L-arginine: NO precursor
[¡]L-NAME: NG-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor
[§]OT: Oxytocin

Authors' Contributions

M.Z. and M.K. conceived and planned the experiments. H.Z. and B.R. carried out the experiments. A.B., M.Z. and K.M. contributed to the interpretation of the results. M.K took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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